ELSEVIER

Journal of Chromatography A, 959 (2002) 269–279

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Method of intracellular naphthalene-2,3-dicarboxaldehyde derivatization for analysis of amino acids in a single erythrocyte by capillary zone electrophoresis with electrochemical detection

Qian Dong, Xiaolei Wang, Lanlan Zhu, Wenrui Jin*

School of Chemistry and Chemical Engineering, and State Key Laboratory of Microbial Technology, Shandong University, Jinan 250100, China

Received 21 January 2002; received in revised form 26 March 2002; accepted 11 April 2002

Abstract

A novel method of intracellular derivatization was developed. In this method, the derivatization reagents [naphthalene-2,3-dicarboxaldehyde (NDA) and CN^-] were introduced into living cells by electroporation for the derivatization reaction. After completion of derivatization reaction in cells, a single cell was drawn into the capillary tip by electroosmotic flow. Then the lysing solution was introduced into the capillary by diffusion. Once the individual cell was lysed, the derivatized amino acids in the individual cell were separated by capillary zone electrophoresis and detected by end-column amperometric detection at the outlet of the capillary. This method of intracellular NDA derivatization confined the analytes and the derivatization reagents to the volume of a single cell expanded. For an 8-μm erythrocyte, the contents were diluted by a factor of only ca. 1.6. The method was used to determination of amino acids in single erythrocytes. Six amino acids were identified and quantified. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Derivatization, electrophoresis; Cells; Erythrocytes; Naphthalene-2,3-dicarboxaldehyde; Amino acids

1. Introduction

Capillary electrophoresis (CE) has come to be a useful and powerful separation technique for the analysis of chemical species [1–3]. CE has many inherent features of its operation suitable for analysis of single cells such as extremely small sample size, high separation speed and efficiency, biocompatible environments and low cost. It has been applied to the determination of the chemical contents of single cells [4–17]. Electrochemical detection (ED) has been an important detection mode for analysis of single cell. It offers a high sensitivity and selectivity for analytes that are electroactive. CE–ED has been used to detect electroactive neurotransmitters in single whole neurons [7], in cytoplasm injections from snail neurons [4,6], in single human lymphocytes [13], and in single sympathetic nerve cells [15]. Glutathione in single human erythrocytes [16], and in individual mouse peritoneal macrophages [17] has also been detected in our laboratory.

Since many species such as most amino acids found in single cells are not natively electroactive, methods must be developed to detect these species. Covalent labeling with an electroactive tag is an attractive method for extending the high sensitivity and selectivity of electrochemical detection. Usually,

*Corresponding author. Fax: +86-531-856-5167.
E-mail address: wenrujin@jn-public.sd.cninfo.net (W. Jin).

0021-9673/02/$ – see front matter © 2002 Elsevier Science B.V. All rights reserved.
PII: S0021-9673(02)00440-5

chemical derivatization is used for determination of the contents in single cells by CE with laser-induced fluorescence (LIF) detection or ED. Four approaches have been used successfully for derivatization of the contents of single cells. The first one can be called the pre-column derivatization scheme, in which single cells physically isolated and placed in a 200-nl microvials is lysed and then derivatized. A portion of the cell lysed and derivatized with the final volume ranged from 20 to 30 nl is injected into the separation capillary [5,18,19]. In the second one, living cells are derivatized by incubating with a derivatization reagent, to which the cell membrane is permeable. Then the whole cell is drawn into the separation capillary and lysed on the front end of the capillary [8]. The third one is an on-column derivatization scheme [20,21]. In this method, the front end of the separation capillary is used as a derivatization chamber, where the cell and then the lysing/derivatizing buffer are introduced by electromigration and mixed. After completion of the derivatization reaction in the front end of the capillary, the derivatized analytes are separated and detected. In this method, the contents of a 20-μm diameter cell are diluted by a factor of approximately 100 during the on-column derivatization. In the last method a post-column reactor for derivatization is developed [22]. It should be noted that the critical problem is minimizing dilution of the contents of a single cell during the derivatization in order to maintain favorable kinetics for the labeling reaction and avoiding diluting the analytes that are already present at trace levels. It seems the second method is better, because the derivatization reaction proceeds inside the cell. However, it cannot be used for those derivatization reagents to which the cell membrane is not permeable.

Sensitive and selective methods for the detection of amino acids are of increasing importance, especially in analysis of single cells. However, most native amino acids have no electroactivity. Naphthalene-2,3-dicarboxaldehyde (NDA) can react with primary amines in the presence of cyanide to produce electroactive cyano[*f*]benzoisoidole (CBI) [23]. The electroactivity of NDA derivatives has been utilized to the analysis of amino acids in a single neuron from the *Helix aspersa* by high-performance liquid chromatography with pre-column derivatization and ED [24], and in a giant dopamine neuron of the snail *P. corneus* (approximately 75 μm in size) by capillary zone electrophoresis (CZE) with on-column derivatization and ED [21], in which five amino acids have been quantitated. Although CZE with on-column derivatization and ED has been also attempted to analysis of amino acids in single cultured pheochromocytoma cells (PC12) with a diameter of 10–15 μm, no amino acids were detected. This is probably due to the dilution of the contents of a single cell during the derivatization. The fluorescent properties of NDA derivatives have also been exploited to determine amino acids in PC12 by CZE with LIF detection [20], in which five amino acids have been quantitated. This is not surprising, because the sensitivity of LIF detection is better than that of ED. Erythrocytes are very small human cells with a diameter of 8 μm and a volume of 87 fl [8]. Up to now, separation and determination of amino acids in a single erythrocyte by CZE has not been reported, especially using ED.

In order to detect amino acids in such a small cell by CE with ED, one way still is by using chemical derivatization to label them with NDA in the presence of cyanide. Minimizing dilution of amino acids in single cells during the derivatization and the lysis still is a critical step. We attempted the second method for the derivatization mentioned above, i.e., living erythrocytes were derivatized by incubating with derivatization reagents. Unfortunately, the cell membrane is not permeable for NDA. In this work, we developed a new method of intracellular NDA derivatization. In this method, NDA and CN^- were introduced into living cells by electroporation for the derivatization reaction. After completion of the derivatization reaction, a whole erythrocyte was drawn into the front of the separation capillary by electroosmotic flow. Then the lysing solution was introduced into the capillary by diffusion. Once the individual erythrocyte was lysed, the derivatized amino acids from individual erythrocytes were separated by CZE and detected by ED at the outlet of the capillary. This method of intracellular NDA derivatization confined the analytes and the derivatization reagents to a volume of cell (87 fl for erythrocytes), significant minimizing dilution.

2. Experimental

2.1. Electroporation equipment

The electroporation equipment used here was the same as in our previous work [25]. Briefly, this generator mainly included two parts: charge and discharge parts. The charge part comprised an alternating current power supply, a selenium rectifier, two electrolyte filter capacitors, a potentiometer, and an electrolyte capacitor (which also as the power supply of the discharge part). The discharge part contained the electrolyte capacitor and an electroporation cell. The electroporation cell mainly included two parallel platinum plate electrodes. The voltage of the electrolyte capacitor charged could be adjusted by using the potentiometer. The capacitor released its charge through the electroporation cell.

2.2. CZE system

Details of the CZE separation system used in this work was similar to our description previously [26]. Briefly, the apparatus consisted of a fused-silica capillary with dimensions of 20 μm I.D.×375 μm O.D. (Yongnian Optical Conductive Fiber Plant, Yongnian, China), which was cut to a length of about 40 or 70 cm and placed between two buffer reservoirs. A high voltage was applied at the injection end, while the reservoir containing the electrochemical detection cell was held at ground potential. Separation was carried out at an applied voltage of 18 kV generated by a high-voltage power supply (Model 9323-HVPS, Beijing Institute of New Technique, Beijing, China). The electrochemical detection at a constant potential with CZE was performed using the electrochemical approach with an electrochemical analyzer (Model CHI800, CH Instruments, Austin, TX, USA). The detection cell and detector were housed in a Faraday cage in order to minimize the interference from noise of external sources. Electrochemical detection was carried out with a three-electrode system. It consisted of a carbon fiber microdisk bundle electrode as the working electrode, a coiled Pt wire as the auxiliary electrode (which also served as a ground for the separation potential) and a saturated calomel electrode (SCE) as the reference electrode. The arrangement of the electrochemical detection cell was previously illustrated in detail [26]. The carbon fiber microdisk bundle electrodes used here were the same as those in Ref. [27].

2.3. Cell preparation

About 0.4 ml human blood with anticoagulant from a normal adult was collected in a 1.5-ml micro-centrifuge tube and centrifuged at 191 *g* for 5 min to separate erythrocytes. Then the supernatant liquid was removed. The erythrocytes remained was about 0.2 ml. In order to wash the erythrocytes, a 6-fold phosphate-buffered saline (PBS) was added into the micro-centrifuge tube. After vibrating lightly, the mixture was centrifuged, and then the supernatant liquid was removed. This step was repeated over five times until the supernatant was clear and transparent. When 0.01 mol/l CN^- of 10 μl and then 0.01 mol/l NDA of 10 μl were added into the supernatant liquid of 80 μl for the last wash, no amino acids was detected. This means that the supernatant liquid around erythrocytes cannot interfere with the determination of amino acids in single erythrocytes. After the supernatant liquid was removed, the erythrocyte solution was obtained with a concentration of 3.91×10^6 cells/μl.

2.4. Preparation and treatment of hemolysate

The amount of cells in the erythrocyte solution was counted using a hemocytometer (Shanghai Medical Optical Instrument Plant, Shanghai, China). Then the erythrocyte solution of 150 μl were collected in a 10-ml centrifuge tube and mixed with 1.25×10^{-2} mol/l borax–3.13×10^{-3} mol/l NaOH of 1.5 ml. When the erythrocytes were lysed, 5% (w/v) sulfosalicylic acid of 400 μl was added, in order to precipitate the protein in the erythrocytes. The mixture was centrifuged at 430 *g* for 15 min. The supernatant liquid was put in a micro-centrifuge tube of 1 ml and adjusted to pH 9.5 with NaOH solution. The solution was stored at 4 °C.

2.5. Pre-column derivatization of amino acids

Pre-column derivatization of standard solutions

and hemolysate samples for the purpose of determining electrophoretic peak identities and quantitation was performed in 1.5-ml centrifuge tubes for the standard solutions and 0.5-ml centrifuge tubes for the hemolysate samples. For the standard solutions an appropriate volume of the stock solutions of amino acids was diluted in a borate buffer of pH 9.48. NaCN was added and then NDA was added. The final concentrations of both NaCN and NDA were 3.0×10^{-4} mol/l. For the hemolysate samples 50 μl hemolysate were mixed with 120 μl running buffer solution; 0.01 mol/l NaCN of 6 μl was added and then 0.01 mol/l NDA of 6 μl was added. The erythrocyte concentration in this solution is 7.9×10^{4} cell/μl All solutions was reacted for at least 30 min before injecting to the capillary.

2.6. CZE operation

Before each run, the capillaries were flushed with 0.1 mol/l NaOH for 5 min, and then with water and the corresponding running buffer for 5 min, respectively, by means of a syringe. The carbon fiber microdisk bundle electrode cemented onto a microscope slide has to be aligned with the detection end of the capillary under a microscope. Then a voltage of 18 kV was applied across the capillary and the detection potential was applied at the working electrode. After the electroosmotic flow reached a constant value (after about 10–20 min), the electrokinetic injection of the standard solutions or the hemolysates or the single cells was carried out. After the analytes or the single cells were injected into the capillary, the capillary was carefully moved from the solutions derivatized or the erythrocyte suspension into a reservoir of 1 ml containing a CZE running buffer. Then the separation voltage of 18 kV was applied and the electropherogram was recorded.

2.7. Cell electroporation and intracellular derivatization

One μl erythrocyte solution was mixed with 0.5% (w/v) trypan blue solution of 60 μl; 0.01 mol/l NDA of 20 μl and then 0.01 mol/l NaCN of 20 μl were added into the solution, respectively. Thus, the erythrocyte suspension was obtained. The erythrocyte suspension was dropped into the place between the two platinum electrodes of the electroporation cell, which had been put on the platform of an inverted biological microscope. Then, the erythrocytes in the suspension were electroporated according to literature [25]. Since the erythrocytes electroporated were blue, they could been monitored under the inverted microscope. Finally, the erythrocyte suspension was taken out from the eletroporation cell and put into a 0.5-ml micro-vessel. Since the erythrocyte suspension contained trypan blue, NDA and NaCN during the electroporation process, they still were in the suspension after electroporation. These reagents in the erythrocyte suspension were washed down by PBS before injecting single erythrocytes into the capillary. The erythrocytes were derivatized inside cells during washing erythrocytes.

2.8. Injection and lysis of whole cell

In order to inject the single erythrocytes introduced NDA and CN^{-} by electroporation into the capillary, the erythrocyte suspension must be transferred directly into the injection end of the separation capillary under the field of view of an inverted biological microscope. A droplet of the erythrocyte suspension of 5 μl was, therefore, placed on a clean microscope slide. After the microscope slide was placed on the inverted biological microscope with a magnification of ×400, the injection end of the capillary filled with electrophoresis buffer was gently immersed in the droplet under the guidance of a three-dimensional micro-manipulator. In order to see the opening of the injection end, a ca. 5-mm section of the polyimide coating at the injection end of the capillary was removed by burning before use. A platinum wire was placed in the erythrocyte suspension to serve as the electrophoresis anode. As soon as the erythrocyte was drifting towards the injection end, an injection voltage of 0.7 kV was applied to draw the whole cell into the capillary tip by electroosmotic flow as observed under the microscope. The erythrocyte was adsorbed on the wall of the capillary 25–50 μm away from its tip. The entire process of cell injection typically took ca. 30 s. Then the capillary was gently moved from the erythrocyte suspension into a droplet of 0.1 mol/l NaOH as the cell lysis solution. The NaOH solution was allowed to diffuse into the capillary tip and around the cell.

The individual erythrocyte was lysed within 10 s. After that, the capillary was manipulated up, out of the NaOH, and immersed into the CZE running buffer solution. Then the separation voltage of 18 kV was applied and the electropherogram was recorded.

2.9. Reagents and solutions

All amino acids (chromatographic grade) and sodium cyanide (chemically pure grade) were obtained from Shanghai Biochemical Reagents Company (Shanghai, China). NDA (content 99%) was purchased from Sigma (St. Louis, MO, USA). A 1.00×10^{-2} mol/l stock solution of amino acids was prepared by dissolving appropriate amounts of amino acids in water. A 1.00×10^{-2} mol/l NDA solution was prepared in acetonitrile and stored in the dark. Sodium cyanide solution (0.10 mol/l) was prepared in water. The borate buffer solutions were made by dissolving an appropriate amount of borax in water and then adjusting the pH of the solutions to the desired pH by addition of NaOH. A 5% (w/v) sulfosalicylic acid was made by dissolving an appropriate amount of sulfosalicylic acid in water. A 0.5% (w/v) trypan blue stock solution was prepared by dissolving an appropriate amount of trypan blue (Sigma) in water. Dilute solutions were obtained by serial dilution of the stock solution with PBS. The PBS consisted of 0.135 mol/l NaCl and 0.02 mol/l NaH_2PO_4–NaOH (pH 7.4). All reagents were of analytical grade except for amino acids, sodium cyanide, trypan blue and NDA. All solutions were prepared with double distilled water and stored at 4 °C.

3. Results and discussion

3.1. Detection of amino acids with pre-column derivatization

We found that 19 amino acids except proline can be derivatized with NDA in the presence of cyanide. The optimum derivatization reaction time is longer than 15 min at room temperature. Their derivative can be oxidized at the carbon fiber bundle electrode in borate buffers. NDA and cyanide in the buffer cannot disturb the detection of amino acids. Fig. 1 shows the electropherogram of the mixture containing 18 amino acids and glutathione (GSH) derivatized by NDA and CN^- using the separation voltage of 18 kV and the detection potential of 1.0 V versus SCE in 1.25×10^{-2} mol/l broax–3.13×10^{-3} mol/l NaOH–3% acetonitrile. In this electropherogram, the concentration ratios between amino acids are close to their concentration ratio in erythrocytes reported in literature [28]. From this electropherogram, it can be found that nine amino acids (Arg, Lys Orn, Tyr, Ser, Ala, Gly, Glu, Asp) can be separated well in the presence of other amino acids.

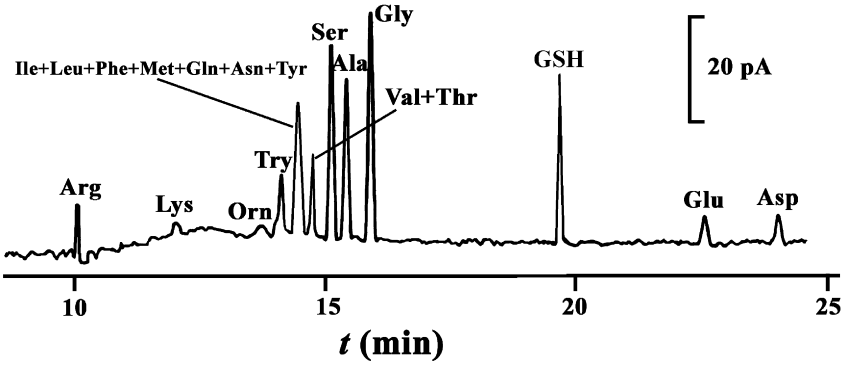


Fig. 1. Electropherogram of 18 amino acids and GSH derivatized by NDA and CN^-: 1.25×10^{-2} mol/l borax–3.13×10^{-3} mol/l NaOH–3% acetonitrile; 6.00×10^{-8} mol/l Met, 1.50×10^{-7} mol/l Ile, Phe and Tyr, 3.00×10^{-7} mol/l Leu, Asn and Thr, 1.00×10^{-6} mol/l Arg, Lys, Orn, Tyr, Gln and Val, 2.50×10^{-6} mol/l Ser, Ala and Gly, 5.00×10^{-6} mol/l Glu and Asp, 5.00×10^{-6} mol/l GSH; 3.00×10^{-4} mol/l NDA; 3.00×10^{-4} mol/l CN^-. Capillary, 70 cm×20 μm I.D.; injection, 3.4 kV for 5 s; separation voltage, 18 kV; detection potential, 1.0 V (versus SCE); the area of carbon fiber bundle microelectrode, 3.5×10^{-4} mm^2.

Ile, Leu, Phe, Met, Gln, Asn and Tyr overlap in one peak, and Val overlaps Thr. Arg has the shortest migration time, t_m, and Asp has the longest t_m. In addition, GSH has a whole peak between Gly and Glu. It cannot interfere with the identification and quantification of the amino acids. An investigation has been performed in order to determine the limits of detection (LODs) for the amino acids identified in the erythrocyte. The results are shown in Table 1. The concentration LODs ranged from1.5×10^{-7} mol/l for Arg, the earliest eluting of the group, to 8.8×10^{-7} mol/l for Asp, the latest eluting of the group. According to the volume calculated theoretically, the mass LODs ranged from 100 amol for Gly to 290 amol for Asp. Overall the peaks of their derivatives are very narrow, exhibiting efficiencies of 48 000–344 000 theoretical plates. The linear relationship exists between the peak current detected and the concentration of amino acids in the range of LOD of each amino acid to at least 1.00×10^{-5} mol/l, respectively.

3.2. Determination of amino acids in hemolysates

A typical electropherogram of the hemolysate without the derivatization with NDA and CN^- is shown in Fig. 2, curve 1 using a capillary of 70 cm length. There is only one electrophoretic peak eluting at ca. 10 min. Tyr, Try, His and Met are somewhat electroactive. When they were derivatized with NDA and CN^-, the peaks of their derivatives appeared around 14 min and the peak at ca. 10 min was still present. This means that the four amino acids were not responsible for the peak at ca. 10 min and the substance concerning the peak was not derivatized. The other amino acids are electroinactive. Therefore, this peak at ca. 10 min is not the peak of amino acids. The electropherogram of hemolysate after derivatization with NDA and CN^- is shown in Fig. 2, curve 2. Ten electrophoretic peaks are obtained. These peaks in the electropherogram except for peaks 3 and 10 have been identified on the basis of their electromigration mobilities and spiking corresponding standard solutions of amino acids. In order to confirm these peaks, the electropherogram of 18 amino acids determined by CZE–ED under the same conditions is shown in Fig. 2, curve 3. In this electropherogram, the concentration ratios between amino acids are close to their concentration ratios in erythrocytes reported in the literature [28]. By comparing Fig. 2, curve 2 with curve 3, it can be found that t_m of the peaks 1, 2, 5, 6, 7, 8 and 9 in the hemolysate is the same as t_m of the peak of Arg, Tyr, Ser, Ala, Gly, Glu and Asp, respectively. Peak 1 (the peak of Arg) also overlaps the peak obtained without derivatization shown in Fig. 2, curve 1, because they have the same t_m. However, peak 1 is higher than the peak without derivatization. Therefore, peak 1 consists of the peak of Arg and the peak of background in the hemolysate. After 1.00×10^{-5} mol/l Tyr, Ser, Ala, Gly, Glu and Asp are added into the hemolysate, respectively, and their derivatization reaction with NDA and CN^- is accomplished, the peak current of peaks 2, 5, 6, 7, 8 and 9 increases, respectively. Therefore, peaks 1, 2, 5, 6, 7, 8 and 9 can be considered as the peaks of Arg, Tyr, Ser, Ala, Gly, Glu and Asp. Now we analyze the other three peaks (peaks 3, 4 and 10). By comparing Fig. 2, curve 2 with curve 3, it can be found that identifying peak 3 is difficult, because its migration time is the same as the peak of Ile, Leu, Phe, Met, Gln, Asn and Tyr, which cannot be separated under the present conditions. Peak 4 should be the peaks of both Val and Thr or one of the both peaks. In order to affirm peak 4, 5.00×10^{-6} mol/l Val and Thr are added in the hemolysate, respectively. After their derivatization reaction with NDA and CN^- is accomplished, the peak current of peak 4 increases and does not widen when Val is added. However, when Thr is added in the hemolysate, peak 4 broadens and its

Table 1
Limit of detection for some standard amino acids by capillary zone electrophoresis with electrochemical detection

Amino acids	Detection limit of concentration (mol/l)	Detection limit of mass (amol)
Arg	1.5×10^{-7}	123
Tyr	2.3×10^{-7}	132
Val	3.4×10^{-7}	186
Ser	2.6×10^{-7}	138
Ala	2.4×10^{-7}	123
Gly	1.9×10^{-7}	100
Glu	6.6×10^{-7}	232
Asp	8.8×10^{-7}	290

Forty-cm capillary. Conditions as in Fig. 1.

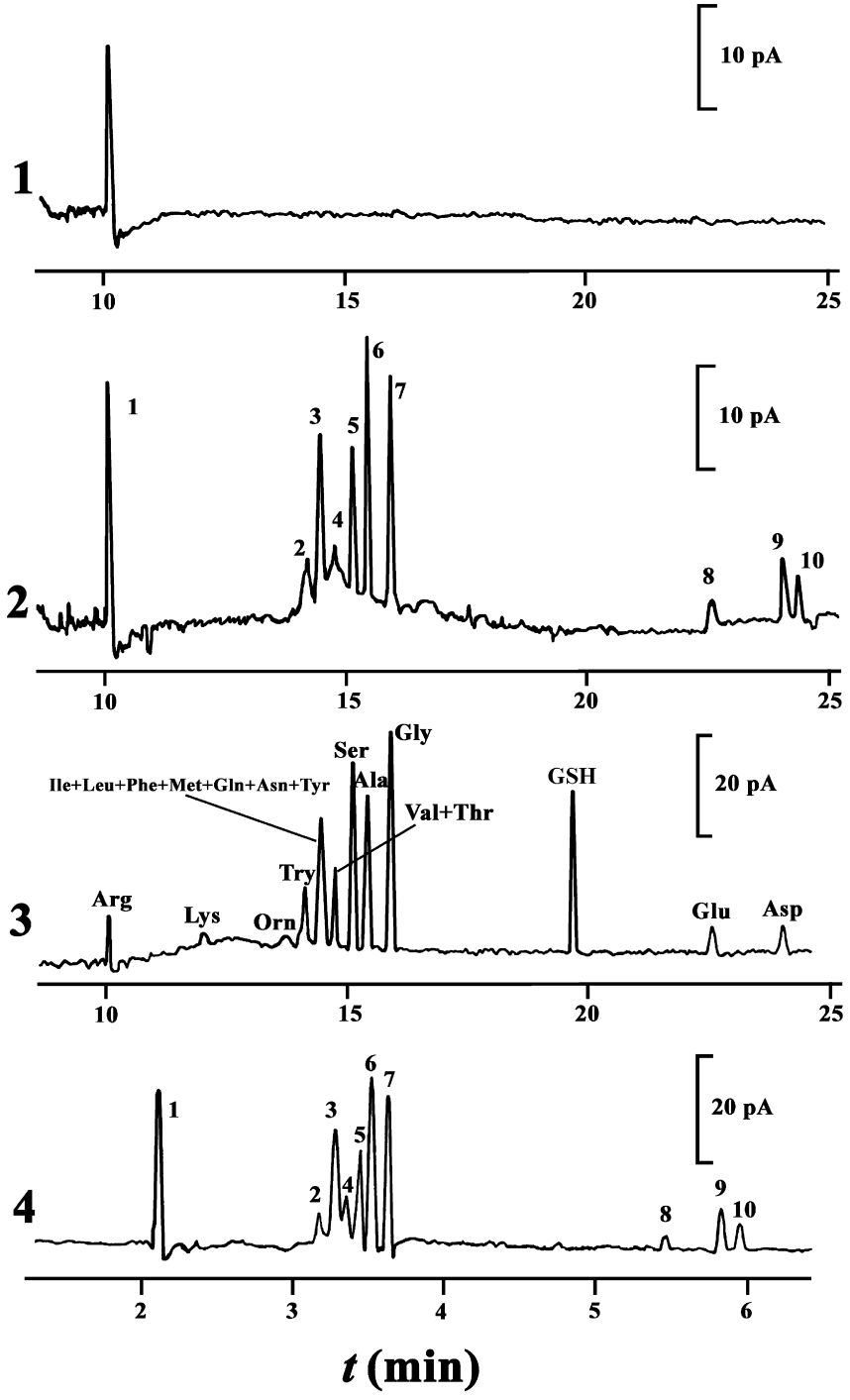
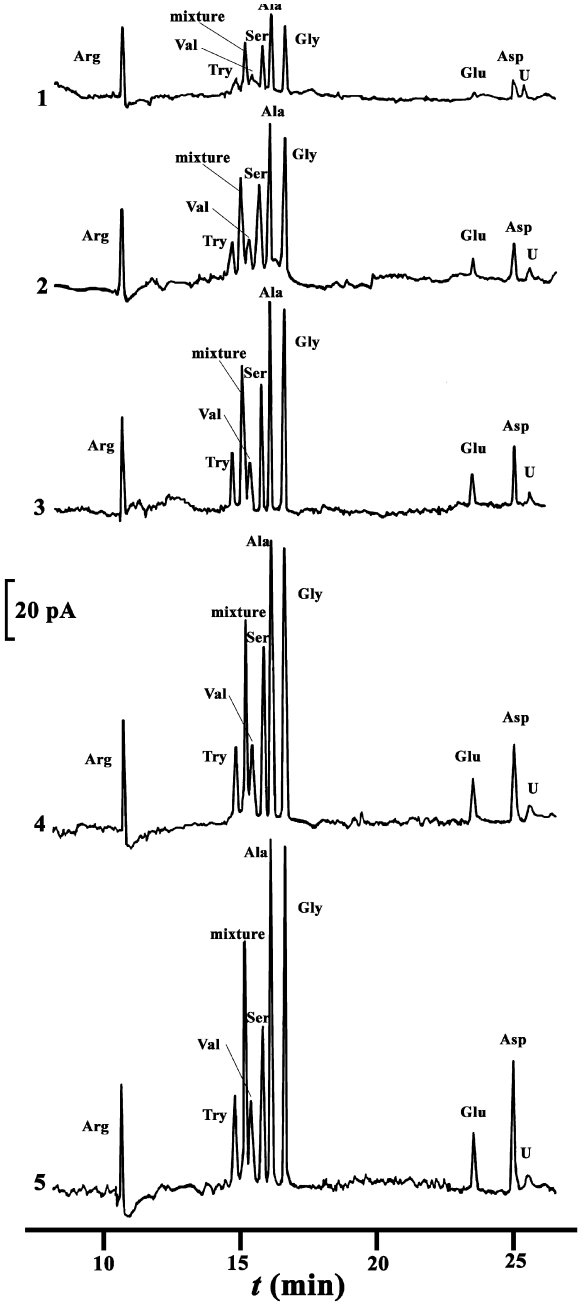


Fig. 2. Electropherograms of hemolysate without and with NDA derivatization and standard solution of 18 amino acids with NDA derivatization. (1) Hemolysate without derivatization; (2,4) hemolysate with derivatization; (3) standard solution of amino acids with derivatization. (1–3) 70-cm capillary; (4) 40-cm capillary. Other conditions as in Fig. 1.

peak current does not increase. Therefore, peak 4 is the peak of Val. Peak 10 has not yet been identified. When a capillary of 40 cm length was used, these peaks still can be separated well, though resolution reduces somewhat (see Fig. 2, curve 4).



In order to quantify the concentration of amino acids in the hemolysates, the standard addition method was used. Moreover, because the peak of Arg after derivatization overlaps the peak of the background, the peak current of the background should be eliminated when the concentration of Arg in the hemolysates is measured. The electropherograms of a hemolysate sample without and with the standard solution of amino acids are shown in Fig. 3. The mean concentrations of these amino acids are 6.07×10^{-7} mol/l for Arg, 4.28×10^{-7} mol/l for Tyr, 4.62×10^{-7} mol/l for Val, 1.39×10^{-6} mol/l for Ser, 1.76×10^{-6} mol/l for Ala, 1.75×10^{-6} mol/l for Gly, 1.32×10^{-6} mol/l for Glu and 2.55×10^{-6} mol/l for Asp. Since the cell concentration in the hemolysate sample is 7.86×10^{4} cell/μl, the mean masses of amino acids in a single erythrocyte can be calculated to be 7.72 amol for Arg, 5.45 amol for Tyr, 5.88 amol for Val, 17.7 amol for Ser, 22.4 amol for Ala, 22.3 amol for Gly, 16.8 amol for Glu and 32.4 amol for Asp. If the mean volume of 87 fl [8] for a single erythrocyte is used, the mean concentrations of amino acids in a single erythrocyte can be obtained to be 8.87×10^{-5} mol/l for Arg, 6.26×10^{-5} mol/l for Tyr, 6.77×10^{-5} mol/l for Val, 2.03×10^{-4} mol/l for Ser, 2.75×10^{-4} mol/l for Ala, 2.56×10^{-4} mol/l for Gly, 1.93×10^{-4} mol/l for Glu and 3.72×10^{-4} mol/l for Asp. The recoveries of the method for the eight amino acids are between 94 and 108%. In the

Fig. 3. Electropherograms of a hemolysate sample without and with standard solution of amino acids after NDA derivatization. (1) Sample. (2) (1)+8.06×10^{-7} mol/l Arg, 5.38×10^{-7} mol/l Tyr and Val, 1.35×10^{-6} mol/l Ser, Ala and Gly, 2.69×10^{-6} mol/l Glu and Asp, 2.00×10^{-7} mol/l Lys, Orn and Gln, 3.22×10^{-8} mol/l Met, 8.06×10^{-8} mol/l Ile, Phe and Tyr, 1.61×10^{-7} mol/l Leu and Asn. (3) (1)+1.61×10^{-6} mol/l Arg, 1.08×10^{-6} mol/l Tyr and Val, 2.69×10^{-6} mol/l Ser, Ala and Gly, 5.38×10^{-6} mol/l Glu and Asp, 4.00×10^{-7} mol/l Lys, Orn and Gln, 6.44×10^{-8} mol/l Met, 1.61×10^{-7} mol/l Ile, Phe and Tyr, 3.22×10^{-7} mol/l Leu and Asn. (4) +2.42×10^{-6} mol/l Arg, 1.61×10^{-6} mol/l Ty and Val, 4.03×10^{-6} mol/l Ser, Ala and Gly, 8.06×10^{-6} mol/l Glu and Asp, 6.00×10^{-7} mol/l Lys, Orn and Gln, 9.66×10^{-8} mol/l Met, 2.42×10^{-7} mol/l Ile, Phe and Tyr, 4.83×10^{-7} mol/l Leu and Asn. (5) (1)+3.23×10^{-6} mol/l Arg, 2.15×10^{-6} mol/l Tyr and Val, 5.38×10^{-6} mol/l Ser, Ala and Gly, 1.08×10^{-5} mol/l Glu and Asp, 8.00×10^{-7} mol/l Lys, Orn and Gln, 1.29×10^{-7} mol/l Met, 3.23×10^{-7} mol/l Ile, Phe and Tyr, 6.45×10^{-7} mol/l Leu and Asn. Unidentified peak is labeled U. 40 cm capillary. Conditions as in Fig. 1.

literature [28] only the concentrations of five amino acids in a single erythrocyte were reported, they were 3.60×10^{-5} mol/l for Arg, 2.00×10^{-4} mol/l for Val, 1.57×10^{-4} mol/l for Ser, 3.42×10^{-4} mol/l for Ala and 3.51×10^{-4} mol/l for Gly. It can be found that the concentrations of Ser, Ala and Gly in a single erythrocyte determined by the current method are very close to the values reported in literature and the concentrations of Arg and Val somewhat different from the values reported in literature. The very small difference is from different erythrocyte samples.

3.3. Electroporation-introducing NDA and CN^- into erythrocytes

The critical values of reversible electroporation for erythrocytes should be find out in the solution containing PBS, NDA, NaCN and trypan blue as an indicator. Trypan blue is a dyestuff to distinguish cells electroporated reversibly. If the cells are electroporated reversibly, they with blue color will be observed under the microscope. It was founded that erythrocytes could not be dyed by trypan blue, when the cells were incubated in trypan blue solution for 24 h. This means that trypan blue could not be introduced into erythrocytes by pinocytosis or diffusion. However, erythrocytes can be stained by trypan blue after cells are electroporated reversibly. It was found that the optimum conditions for electroporation-introducing the reagents into erythrocytes were 8 kV/cm for the pulse strength, 4 for the pulse number, and 40 μs for the pulse duration. In this case, the electroporation efficiency, defined as the percentage of cell reversibly electroporated among the whole cells, is 90%. The sequence of adding the reagents is very important for analysis of amino acids in single erythrocytes using the derivatization with NDA and CN^-. If NaCN is added in the erythrocyte suspension before NDA is added, erythrocytes shrink and amino acids will go into the erythrocyte suspension from erythrocytes. If NDA is added and then NaCN is added, erythrocytes do not change.

3.4. Analysis of amino acids in single erythrocytes

Usually, cell lysis is accomplished by injecting a plug of lysis solution around the cell in the separation capillary. Lysis of human erythrocytes has been researched in our laboratory. Erythrocytes can easily be lysed in CZE running buffers, but erythrocytes introduced NDA and CN^- by electroporation cannot. It was noted that erythrocytes with NDA and CN^- could not be lysed in buffers of pH 1–10. However, they could be lysed in 0.1 mol/l NaOH. Therefore, After an erythrocyte is injected and adsorbed on the wall of the capillary, the capillary was gently moved from the erythrocyte suspension into a droplet of 0.1 mol/l NaOH as the cell lysis solution. The NaOH solution is allowed to diffuse into the capillary tip and around the cell. The individual erythrocyte is lysed within 10 s.

Since erythrocytes are suspended in PBS, PBS is injected into the separation capillary with an erythrocyte during injecting the cell. Therefore, the behavior of PBS should be observed first. The electropherogram of PBS is shown in Fig. 4, curve 1. From the electropherogram, it can be found that there are two peaks on the electropherogram of PBS. One (peak 1) is high and narrow and the other (peak 2) is low and wide. As compared to the electropherogram of the standard solution of amino acids shown in Fig. 4, curve 2, the two peaks do not interfere with the identification of amino acids. A typical electropherogram of the contents of an individual erythrocyte obtained using intracellular derivatization with NDA and CN^- is shown in Fig. 4, curve 3. Four small peaks eluting within 3–4 min following blank peaks 1 and 2. In order to distinguish the four electrophoretic peaks clearly, they are magnified five times and shown in Fig. 4, curve 4. Identification of the peaks detected is possible through comparison with the electropherogram shown in curve 2, indicating the peaks are Ser, Gly and Ala. Identification of peak 3 is difficult, because many amino acids have the same migration time as that of peak 3.

Due to the small volumes and low analyte levels, the internal standard of individual cells was difficult to analyze [9]. The reproducible peak currents, together with the large linear dynamic range for standard amino acids made it suitable to use external standardization for the quantification of amino acids in an erythrocyte. In order to measure the amounts of amino acids in individual erythrocytes accurately, the contents of amino acids in individual erythrocytes

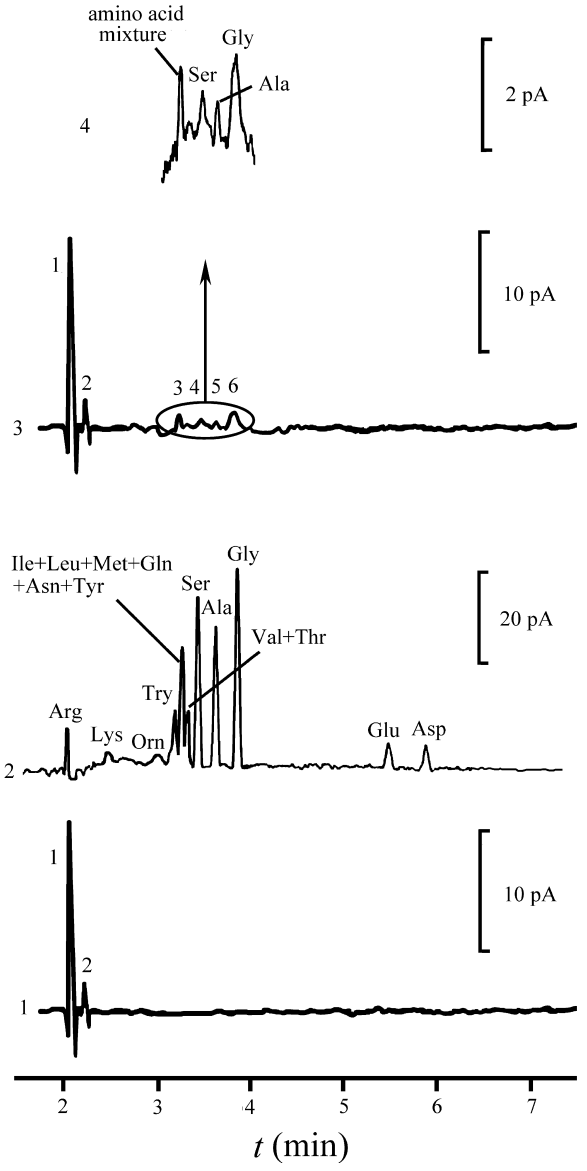


Fig. 4. (1) Electropherogram of PBS. (2) Electropherogram of standard amino acids with NDA derivatization. (3) Electropherogram of an erythrocyte with intracellular NDA derivatization. (4) Five times of a part of curve 3. Forty-cm capillary. Conditions as in Fig. 1.

are quantified by comparison of the peak current against those of standard amino acids injected after each cell run. We determined 116 cells. However, the amino acids only in 16 cells were detected, because the contents of amino acids in these cells are higher than their detection limits. Quantitation of the components present in the erythrocytes is shown in Table 2. Although the contents of the six amino acids could be obtained only in 16 of 116 cells, the method with intracellular derivatization using electroporation, after all, could determine the amino acids in an erythrocyte. This is because the volume of a cell limits dilution of reagents and the cell contents during derivatization. Geometry of an erythrocyte is like a ''cake'' with a diameter of 8 μm and a volume of 87 fl. Th height of the ''cake'' can be calculate to be 1.73 μm. After derivatization, a diameter of the erythrocyte changes to 10 μm. In this case, the volume of the erythrocyte can be calculate to be 136 fl. The contents of an 8-μm diameter erythrocyte are diluted by a factor of approximately 1.6 during intracellular derivatization. This method represents a significant reduction in dilution compared to on-column derivatization with a factor of ca. 100 [20].

Table 2
Contents of amino acids determined in 16 individual erythrocytes (amol)

Cells	Tyr	Ser	Ala	Gly	Glu	Asp
1	nd	nd	nd	159	232	nd
2	nd	nd	nd	207	380	nd
3	132	nd	nd	100	nd	nd
4	nd	nd	133	nd	349	nd
5	nd	138	128	nd	305	nd
6	nd	nd	272	nd	nd	290
7	nd	nd	nd	nd	237	296
8	290	nd	nd	nd	nd	nd
9	nd	216	279	nd	nd	nd
10	nd	nd	392	nd	310	nd
11	216	nd	nd	319	nd	nd
12	338	nd	nd	342	nd	nd
13	298	nd	nd	448	nd	nd
14	nd	nd	nd	213	324	nd
15	nd	231	123	445	nd	nd
16	251	nd	174	157	nd	nd

Forty-cm capillary. nd, not detected. Conditions as in Fig. 1.

4. Conclusion

Electroporation can reversibly introduce the derivatization reagents, which cannot naturally permeate cell membrane, into cells, where contents are derivatized with the reagents. The intracellular derivatization hardly dilute the contents of cells. There-

fore, the method makes CZE with electrochemical detection to have the ability of determining electroinactive species such as amino acids in small single cells in size such as erythrocytes. In principle, the method of introducing reagents into cells also should be suitable for use in other fields, in which the reactions of contents of cells with the reagents are investigated in living cells. Further experiments will focus on identification and quantitation of even more amino acids and extending the method.

Acknowledgements

This project was supported by the National Natural Science Foundation of China, the Natural Science Foundation of Shandong Province and State Key Laboratory of Electroanalytical Chemistry, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences.

References

[1] J.W. Jorgenson, K.D. Lukacs, Anal. Chem. 53 (1981) 1298.
[2] C.A. Monnig, R.T. Kennedy, Anal. Chem. 66 (1994) 280R.
[3] H.A. Bardelmeijer, H. Lingeman, C. de Ruiter, W.J.M. Underberg, J. Chromatogr. A 807 (1998) 3.
[4] R.A. Wallingford, A.G. Ewing, Anal. Chem. 60 (1988) 1972.
[5] R.T. Kennedy, M.D. Oates, B.R. Copper, B. Nickerson, J.W. Jorgenson, Science 246 (1989) 57.
[6] T.M. Olefirowicz, A.G. Ewing, Anal. Chem. 62 (1990) 1872.
[7] T.M. Olefirowicz, A.G. Ewing, Chimia 45 (1991) 106.
[8] B.L. Hogan, E.S. Yeung, Anal. Chem. 64 (1992) 2841.
[9] T.T. Lee, E.S. Yeung, Anal. Chem. 64 (1992) 3045.
[10] Q. Xue, E.S. Yeung, J. Chromatogr. A 661 (1994) 287.
[11] Q. Xue, E.S. Yeung, Anal. Chem. 66 (1994) 1175.
[12] Z. Rosenzwig, E.S. Yeung, Anal. Chem. 66 (1994) 1771.
[13] J. Bergquist, A. Tarkowski, R. Ekman, A.G. Ewing, Proc. Natl. Acad. Sci. USA 91 (1994) 12912.
[14] R.A. Wallingford, A.G. Ewing, Anal. Chem. 59 (1987) 1762.
[15] S. Hu, D. Pang, Z. Wang, J. Cheng, Z. Li, Y. Fan, H. Hu, Chem. J. Chin. Univ. 17 (1996) 1207.
[16] W. Jin, W. Li, Q. Xu, Electrophoresis 21 (2000) 774.
[17] W. Jin, Q. Dong, X. Ye, D. Yu, Anal. Biochem. 285 (2000) 255.
[18] M.D. Oates, B.R. Cooper, J.W. Jorgenson, Anal. Chem. 62 (1990) 1573.
[19] A.M. Hoyt, S.C. Beale Jr., J.P. Larmann, J.W. Jorgenson Jr., J. Microcol. Sep. 5 (1993) 325.
[20] S.D. Gilman, A.G. Ewing, Anal. Chem. 67 (1995) 58.
[21] F.D. Swanek, B.B. Anderson, A.G. Ewing, J. Microcol. Sep. 10 (1998) 185.
[22] S.D. Gilman, A.G. Ewing, Methods Instrum. 2 (1995) 133.
[23] P. de Montigny, J.F. Stobaugh, R.S. Givens, R.G. Carlson, K. Srinivasachar, L.A. Sternson, T. Higuchi, Anal. Chem. 59 (1987) 1096.
[24] M.D. Oatas, B.R. Cooper, J.W. Jorgenson, Anal. Chem. 62 (1990) 573.
[25] Q. Dong, W. Jin, Electrophoresis 22 (2001) 2786.
[26] W. Jin, Q. Weng, J. Wu, Anal. Chim. Acta 342 (1997) 67.
[27] W. Jin, D. Yu, Q. Dong, X. Ye, J. Chromatogr. Sci. 38 (2000) 11.
[28] W.P. Leighton, S. Rosenblatt, J.D. Chanley, J. Chromatogr. 164 (1979) 427.